

Marked-up Version of Amendments:

1.

EXEMPLIFICATION

Example 1

In one example, a cloning vector comprising a bifunctional nucleic acid molecule-encoding sequence is prepared in the following manner: The following oligodeoxynucleotides are synthesized: 1) 5'AACGGCCGCGGCTAGTCCACACACAGAACCGTT3' (SEQ ID NO: 1), the sense strand encoding a vascular endothelial growth factor-binding RNA (Jellinek et al, Biochemistry 1994 33:10450-10456) and 2) the complementary strand 5'AACGGTTCTGTGTGTGTGGACTAGCCGCGGCCGTTTCGA3' (SEQ ID NO: 2) with an additional 3' Hind III compatible sequence. These oligonucleotides are admixed and annealed to each other. A nucleotide sequence including the E. coli beta galactosidase gene is prepared by digesting the pUC19 plasmid (Genbank accession number X02514) with Nar I and Hind III, and isolating the resulting 212 base pair fragment by agarose gel electrophoresis and elution. The annealed oligonucleotide and the pUC 19-derived fragment are ligated using T4 DNA ligase. The resulting molecule is ligated to pSP70 plasmid (Promega, Madison, WI) that has been digested with Cla I and Xho I, exploiting the complementarity of Nar I and Cla I overhangs. The free Xho I end of the plasmid is blunted with Klenow and the plasmid is circularized with T4 DNA ligase. An RNA bifunctional nucleic acid molecule is obtained by subjecting the plasmid to standard in vitro transcription procedures using SP6 RNA polymerase.

Example 2

In another example, the following oligodeoxynucleotides are synthesized:

1)) 5'CGCGAACGGCCGCGGCTAGTCCACACACAGAACCGTT3' (SEQ ID NO: 3), the sense strand encoding a vascular endothelial growth factor-binding RNA (Jellinek et al, Biochemistry 1994 33:10450-10456) with an additional 5' Hae II compatible site and 2) the complementary strand 5'AACGGTTCTGTGTGTGTGGACTAGCCGCGGCCGTTTCGA3' (SEQ ID NO: 4). These oligonucleotides are admixed and annealed to each other. A nucleotide sequence including the E. coli beta galactosidase gene is prepared by digesting the pUC19 plasmid (Genbank accession number X02514) with Hae I and Hind III, and isolating the resulting 212 base pair fragment by agarose gel electrophoresis and elution. The annealed oligonucleotide and the pUC 19-derived fragment

2.

Example 5

The aptamer of Example 4 is synthesized with an additional 5'GGGGGGGCCCCCCC3' (SEQ ID NO: 6) at the 5' end and an additional 5'AAAAAAUUUUUUU3' (SEQ ID NO: 6) at the 3' end. The antisense sequence of Example 4 is synthesized with an additional 5'AAAAAAUUUUUUU3' (SEQ ID NO: 6) at the 3' end. The molecules are admixed in a molar ratio of 8 aptamers: 1 antisense for annealing into concatemers.

The resulting bifunctional nucleic acid is useful for treating malignant tumors.

Example 6

A phosphorothioate antisense oligodeoxynucleotide designed to inhibit expression of the reporter protein Enhanced Green Fluorescent Protein (EGFP) was Watson-Crick hybridized to an aptamer with high affinity for the cell surface receptor human L-selectin. This nucleic acid molecule was then incubated with Jurkat cells, a human leukemic T-cell line which naturally expresses human L-selectin, and which, for the purpose of this experiment, had been stably transfected with the pEGFP-n2 expression plasmid encoding EGFP (Clontech Laboratories, Palo Alto, CA). Cells were then analyzed by flow cytometry to determine fluorescence intensities after incubation with aptamer-antisense nucleic acid molecules or with antisense alone.

For this experiment a 32 base mixed-backbone oligodeoxynucleotide with a sequence of: 5'TGGTACCACTCGTTCCCGGATGGATGCTAGAC3' (SEQ ID NO: 7) was purchased from Synthesgen, LLC (Houston, TX) and purified by reverse-phase HPLC. The first 18 bases at the 5' end were Watson-Crick complementary to a region of the pEGFP-n2 plasmid starting three bases before the start codon of the EGFP gene and were linked by phosphorothioate bonds. The final 14 bases at the 3' end of the oligodeoxynucleotide had a phosphodiester backbone and were Watson-Crick complementary to the 5' end of a 79-base single stranded phosphodiester aptamer with high affinity and specificity for human L-selectin (SEQ ID#134 of Parma et al, PCT Publication WO 96/40703). The sequence of this aptamer was 5'CTACCTACGATCTGACTAGCCGGACATGAGCGTTACAAGGTGCTAAACGTAACGT TGCTTACTCTATGTAGTTCC3' (SEQ ID NO: 8) (purchased from Midland Certified Reagent Co. Midland, TX, where it was purified by trityl-selective perfusion HPLC).

3.

5'CTACCTACGATCTGACTAGCCGGACATGAGCGTTACAAGGTGCTAAACGTAACGTTGCTTACTCTATGTAGTTCC3' (SEQ ID NO: 9).

Example 8

A bifunctional nucleic acid is constructed using the aptamer of Example 6 and an antisense molecule consisting of the 14 3' bases of the antisense molecule of Example 6 appended 3' to the phosphorothioate antisense molecule GEM 91, which inhibits the expression of HIV gag and is described in Veal et al, 1998, Antiviral Research 38:63-73. Aptamer and antisense are annealed in PBS at room temperature. The bifunctional nucleic acid molecule can target the antisense to T cells and is useful for treating HIV infection.

Example 9

The reporter gene pEGFP-n2 from Clontech Laboratories is linearized by digestion with the restriction enzyme ApaLI in a non-coding region leaving a 5'sense overhang with the sequence ACGT on one end and an identical overhang on the 5'antisense strand. The following oligodeoxynucleotides are synthesized: 1) 5'TGCAGGGGGGGGGAAGTACATGAGAG3' (SEQ ID NO: 10), which has the first 4 bases at 5' end complementary to the ApaLI overhang, an internal 8 bases which are complementary to the oligomer #2, and the final 15 bases which are

Watson-Crick complementary to the 5' end of a 79 base human L-Selectin aptamer, #3, 2) 5'CCCCCCCC3' which is complementary to 8 bases internal in oligomer #1, and 3) 5'CTACCTACGATCTGACTAGCCGGACATGAGCGTTACAAGGTGCTAAACGTAACGT TGCTTACTCTATGTAGTTCC3' (SEQ ID NO: 11) which is a high affinity aptamer for human L-selectin (SEQ ID#134, Parma et al, PCT Publication WO 96/40703) The first two oligodeoxynucleotides are annealed together by room temperature incubation and then are ligated to the linearized pEGFP-n2 plasmid with T4 DNA ligase. The linearized pEGFP-n2 plasmid now has two linkers ligated to both of its ends which are complementary to a 15 base region of the 5' end of the human L-selectin aptamer. The pEGFP-n2 plasmid is incubated with the aptamer at a 1:2 molar ratio in a buffer consisting of 1mM CaCl₂, 1mM MgCl₂, 125mM NaCl, 5mM KCl, and 20mM HEPES, pH 7.4 for 30 minutes. Wild type Jurkat cells at a concentration of 5 x 10⁵ which endogenously express human L-selectin are then added to the plasmid and aptamer mix, and incubation continues for another 30 minutes. The mix is then equally divided and diluted into duplicate

4.

tubes containing RPMI 1640 with 10% FCS and 100μM chloroquine. After 72 hours cells which have been stably transfected with pEGFP are selected for Neomycin resistance by addition of 1mg/mL of G41 8 antibiotic. Only cells which have stably integrated the neomycin resistance gene included in the pEGFP-n2 plasmid will survive.

A control group consists of cells which receive only linkers ligated to plasmid in a 2:1 molar ratio. For each group of cells a range of final pEGFP-n2 concentrations of 0 through 500 pM up to 1μM is used. The aptamer-plasmid molecule will yield a transfection efficiency at least 20% higher than that of plasmid alone, as measured by number of selected clones.

Example 10

A variation of example 6 would entail using the same human L-selectin aptamer oligodeoxynucleotide (SEQ ID#134, Parma et al, PCT Publication WO 96/40703) with a sequence of:

5'CTACCTACGATCTGACTAGCCGGACATGAGCGTTACAAGGTGCTAAACGTAACGT TGCTTACTCTATGTAGTTCC3' (SEQ ID NO: 11) in conjunction with three distinct mixed backbone antisense molecules. Each antisense molecule will retain the 18 phosphorothioate bases which are complementary to the EGFP start codon region, and will have at least one end with 14 phosphodiester bases complementary to the aptamer molecule. Antisense 1 (A1) has the sequence: 5'TGGTACCACTCGTTCCCGGATGGATGCTAGAC3' (SEQ ID NO: 7) which is identical to the antisense used in example 6 and consists of an EGFP hybridization region in the first 18 bases of the 5' end and an aptamer hybridization region at the 14 bases of the 3' end. A2 has the sequence 5'AGAGTACATCAAGGTGGTACCACTCGTTCCCG3' (SEQ ID NO: 12) in which the first 14 bases at the 5' end are complementary to the first 14 bases at the 3' end of the aptamer molecule, and the last 18 bases at the 3' end comprise the EGFP hybridization region. The A3 molecule contains aptamer hybridization regions at both the 5' and 3' region and maintains the EGFP antisense sequence in the internal 18 phosphorothioate base pairs: 5'AGAGTACATCAAGGTGGTACCACTCGTTCCCGGATGGATGCTAGAC3' (SEQ ID NO: 13).

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Title: Nucleic Acid Compositions and Methods of Introducing Nucleic Acids Into Cells

Page 9 of 9

By varying the molar ratio of A1 and A2 to A3, it is possible to regulate the length of a concatamerized aptamer-antisense chain. For example, aptamer, A1, A2, and A3 can be admixed in a molar ratio 3:1:1:2, so that the nucleic acid molecules thus formed will on average comprise